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Abstract

Nest building consists of a series of motor actions, which are concomitant with activity in regions of the anterior motor pathway, the social behaviour network and the reward circuity in nest building adult male zebra finches (*Taeniopygia guttata*). It is not clear, however, whether this activity is due to nest building, collection and/or manipulation of nest material. To identify which areas of the brain are specifically involved, we used immunohistochemistry to quantify the immediate early gene c-*fos* in male zebra finches that were nest building (Building), birds given a nestbox but could interact only with tied down nest material (Fixed), and birds that were not given a nestbox or nest material (Control). We investigated the following brain regions: the anterior motor pathway (anterior ventral mesopallium (AMV), anterior nidopallium (AN), anterior striatium (ASt)), areas of the social behaviour network (bed nucleus of the stria terminalis, dorsomedial sub division (BSTmd), lateral septum (LS)), the dopaminergic reward circuitry (ventral tegmental area (VTA)) and the cerebellum. We found that there was greater Fos-ir expression in the BSTmd, LS and AMV with increased material deposition; in LS, AMV ASt and folia VI with increased material carrying; in LS, AMV and ASt with increased nest material tucking; and in LS and all folia (except folium VIII) with increased tugging at tied down material. These data confirm a functional role for areas of the anterior motor pathway, social behaviour network and the cerebellum in nest material collection and manipulation by birds.

Abbreviations: AMV, anterior ventral mesopallium; AN, anterior nidopallium; ASt, anterior striatum; BSTmd, bed nucleus of the stria terminalis, dorsomedial subdivision; BSTmv, bed nucleus of the stria terminalis, ventral subdivision; Fos-ir, fos immunoreactivity; LS, lateral septum; VTA, ventral tegmental area.

1. Introduction

Avian reproductive behaviour includes territorial defence, courtship, pairing, nest building, egg laying, incubation, and parental care. Although the neural underpinnings of many of these reproductive behaviours have been well studied (e.g. Heimovics and Riters 2006; Meddle et al. 1999; O'Connell and Hofmann 2011), as have the ultimate causes of nest building (e.g. Hansell 2000; Mainwaring et al. 2014), the neurobiology of nest building has received much less attention to date. Nest building consists of a sequence of actions: a nest site must be located, material is then collected and deposited, and the nest is constructed (Hansell 2000; Walsh et al. 2013). The brain regions involved in nest-building behaviours have typically been quantified by the production of the immediate early gene *c-fos* protein product Fos, as a molecular indicator of neuronal activity (Clayton 2000; Hall et al. 2014; Hall et al. 2015; Heimovics and Riters 2006; Klatt and Goodson 2013; Meddle and Follett 1997). For example, Heimovics and Riters (2006) found that captive adult male European starlings (*Sturnus vulgaris*) with a nestbox in the breeding season had elevated neuronal activity in several areas of the social behaviour neural network, in comparison to males without a nestbox. These social behaviour network regions included the bed nucleus of the stria terminalis, dorsal subdivision (BSTmd) and ventral subdivision (BSTmv), but as nest-building behaviour was not specifically quantified the differences in neuronal activity specifically related to nest building or to courtship or territorial defence could not be disassociated. This is particularly pertinent as activity in the BSTmd and BSTmv, as well as the lateral septum (LS), is associated with territorial defence behaviours in birds (Goodson 2005).

In our previous study (Hall et al. 2014) we examined neuronal activity in the brain during nest building in zebra finches and demonstrated that as nest material pick up and deposit increased, so did the amount of Fos-immunoreactivity (Fos-ir) produced in the

anterior motor pathway, specifically in the anterior ventral mesopallium (AMV), anterior nidopallium (AN), and the anterior striatum (ASt) as well as in the ventral tegmental area (VTA) in the dopaminergic reward circuitry. However, in that study we did not dissociate whether the neural activity resulted from general handling of nest material or was due to nest possession and material collection.

Nest building consists of nest site selection, material collection and often entails fine motor actions of the beak, and for some species the feet, to manipulate material into a small space (e.g. Muth and Healy 2014). As nest building consists of a sequence of organised, discrete motor actions as well as learning (Bailey et al. 2014; Muth and Healy 2014; Thorpe 1956; Tinbergen 1953), and the cerebellum is involved in fine motor control, learning and memory (Middleton and Strick 2000), it seems plausible that the cerebellum plays an integral part in nest construction. Furthermore, as there is evidence that cerebellar foliation increases with nest complexity (Hall et al. 2013), the large variation in cerebellar volume and degree of foliation may provide the neural substrates leading to fine motor control (Butler and Hodos 2005).

The cerebellum can be subdivided into individual folia, which receive different combinations of somatosensory input from different parts of the body; for example, folia I – VI receive afferent somatosensory information originating from neck musculature (Necker 2001). It may then be the case that different folia are involved in different behaviours, for example folia I – VI might be involved in behaviours that require beak movement (e.g. preening, feeding or picking up nest material) and folium IX receives input from neck musculature and the legs (Feenders et al. 2008; Necker 2001).

To determine which of the brain regions previously associated with nest building (Hall et al. 2014) are associated with nest-material selection, collection and handling, we

tested the hypothesis that the cerebellum, anterior motor pathway, social behaviour network and the dopaminergic reward circuitry are specifically involved in the collection and/or handling of nest material in captive male zebra finches. We used three groups of zebra finches: Builders (pairs allowed to build a nest), Fixed (pairs provided with material that was tied down so that the birds could interact with the material but not build a nest), and Controls (pairs that were not provided with material). To identify neuronal activity in zebra finches, we quantified Fos-ir throughout the brain. Given the beak and neck movements required to build a nest (Hansell 2000; 2005), the role of the cerebellum and anterior motor pathway in fine movements, and the Hall et al. (2014) data, we expected Fos-ir expression in the brain regions we examined to increase with increasing handling of material (in or out of a nest).

2. Materials and methods

2.1. Subjects

60 adult zebra finches (30 of each sex) were bred at the University of St Andrews, Scotland, UK. The sample size was chosen based on the numbers of birds required to obtain significance in our previously published studies (Hall et al. 2014; Hall et al. 2015). The birds were housed in single-sex colony cages, maintained on a 14L:10D light:dark cycle, at 19-21°C and 50-65% humidity. All colony and holding cages were lined with wood pellet bedding. Birds had *ad libitum* access to finch seed mix, water, oyster shell grit, cuttlefish bone and a mineral block. Three times a week water was supplemented with calcium and vitamin D3, and food was supplemented with spinach. All experimental procedures were approved by the University of St Andrews Animal Welfare and Ethics Committee.

2.2. Treatment group assignment

Birds had previously been paired (partners were randomly assigned) and had successfully built nests. Birds were re-paired with the same partner and placed in holding cages (50 x 25 x 25 cm) in the same room but were visually isolated from one another.

To ensure all pairs were motivated to build a nest prior to behavioural observations, four pairs were randomly selected, given 240 pieces of 15cm long cotton string (No. 4 Polished Cotton Twine; Rope Source, UK) and left for approximately 16 hours. Following inspection, the next day an experimental cohort was created from the pairs that had begun to build a nest by randomly assigning one pair to each treatment group (Building, Fixed or Control). Pair formation and motivation to build needed to be confirmed before selecting a pair, although it should be noted that this meant that all pairs (including the Control birds) handled material prior to the experiment.

Established pairs were then moved to the test cages (100 x 50 x 50 cm) and left to habituate for approximately 18 hours. This selection procedure continued until there were 10 pairs of birds for each treatment. The test cages were of similar design to the holding cages but to prevent building with wooden pellets, the floor was covered in brown paper. Nest cups were only placed in the cages with Building and Fixed pairs. Control pairs were not provided with a nest cup as we wanted to distinguish between neural activation caused by nest possession and activation caused by nest building and material handling.

2.3. Behavioural observations

On the day following habituation, 30 minutes after lights on, a nest cup was added to the Building and the Fixed treatment cages. Four piles of string made up of 60 pieces (240 pieces/cage) were added to the Building treatment and four sets of 60 pieces of string, with

144 one end tied to the cage bars, were added to the Fixed treatment. By tying one end of the
145 string to the cage bars the birds were able to tug at the string, but not use the material to
146 build a nest. All pairs were digitally recorded using three birdbox cameras (SpyCamera CCTV,
147 Bristol, UK) mounted inside each cage and the video feed was recorded onto a laptop.
148 Sacrifice time for all birds was set for 90 minutes after the male of the Building pair
149 began depositing string in the nest cup, even if the Fixed male had already begun tugging at
150 the tied down string. The birds were monitored via a window in the door of the test room so
151 as not to disturb the birds, and time was recorded when the Building pair made the first
152 deposit of string into the nest cup. If the nest-building male began to build immediately
153 after receiving the string, the sacrifice time was delayed by 15 minutes to avoid Fos-ir being
154 associated with material being delivered to the nest builder's cage. If the Building male did
155 not deposit material in the nest within four hours of the experiment starting, the whole
156 experiment was terminated: the string and nest cups were removed from all cages, and
157 another attempt was made the following day.
158 Behavioural data were only recorded for the first 45 minutes of the experiment.
159 From the video output for the Building and Fixed birds, the occurrence of seven nest-
160 building behaviours were recorded: *depositing* (bird released string into nest), *pick up*
161 (selecting material), *tuck* (bird touched and rearranged material in the nest), *tugging*
162 (pulling on string fixed string), *tugging and hopping* (hopping along cage floor while tugging
163 at fixed string), *tugging and flying* (attempting to fly off with fixed string), and *hopping with*
164 *string*. The duration and number of bouts of birds *flying with string* was also recorded.
165 Example clips of tugging and tucking can be found in the Supplementary material.
166 For all birds *allopreening, drinking, feeding, grooming, hopping, jumping* and
167 *scratching* were also quantified along with the number of bouts and the total duration of

168 birds *flying*. All behaviours were coded using BORIS behavioural analysis software (Friard
169 and Gamba 2016).
170
171 *2.4. Brain tissue collection*
172 90 minutes following initiation of nest-building, pairs of birds were terminally anesthetised
173 (0.2ml sodium pentobarbitone) and the brain was dissected from the skull and fixed by
174 submersion in 4% paraformaldehyde in phosphate-buffered saline (0.1M PBS, pH = 7.4; PFA)
175 for six days at 4°C. Brains were then immersed in 15% sucrose in PFA for 24 hours at 4°C,
176 and then transferred into 30% sucrose in PBS for 24 hours, at 4°C. Brains were then frozen
177 on powdered dry ice, wrapped in foil and stored in labelled plastic bags at -80°C. Samples
178 were then transported on dry ice to the Roslin Institute, University of Edinburgh, Easter
179 Bush, UK were they were stored at -80°C until processing for immunohistochemistry.
180 The cerebellum was separated from the brain and processed separately. The
181 cerebellum was sectioned on a sagittal plane and the forebrain coronally sectioned on a
182 freezing microtome (section thickness = 50μm), and the sections collected in 0.1M PBS. The
183 sections were then stored for 24 hours in PBS at 4°C before immunohistochemical
184 processing for Fos-ir. The coronal forebrain sections were transferred from the PBS into
185 cryoprotectant and then stored at -20°C for 22 months before being sectioned in the same
186 manner as the cerebellum.
187
188 *2.5. Fos immunohistochemistry*
189 Immunohistochemistry was processed in two stages, the cerebellum and forebrain. All birds
190 were processed in the same immunohistochemical run. All sections were processed in
191 Corning netwell baskets and tray system. Sections were washed for 15 minutes, three times,

192 in 0.2% Triton X-100 in 0.1M phosphate buffer (PBS-T) on a shaking platform and then
193 rinsed for 5 minutes in 0.1M PBS. Sections were then incubated for 20 minutes in 0.3% H_2O_2
194 in 0.1M PBS followed by three 10-minute washes in 0.1M PBS-T.

195 Sections were then incubated in 10% Normal Goat Serum (Vector Laboratories) in
196 0.1M PBS-T for 60 minutes to reduce endogenous peroxidase activity and then incubated
197 for 120 minutes at room temperature in 10% Normal Goat Serum in 0.1M PBS-T containing
198 the primary Fos antibody (1:5000; Santa Cruz Biotechnology rabbit polyclonal anti-Fos K-25,
199 sc-253). Incubation continued for approximately 20 hours at 4°C. This antibody has been
200 validated previously for use in zebra finches (Nordeen et al. 2009) and used to identify
201 patterns of neuronal activity associated with nest building in zebra finches (Hall et al. 2014;
202 Hall et al. 2015; Kingsbury et al. 2015; Klatt and Goodson 2013).

203 Any excess unbound antibody was removed by three 10-minute rinses in 0.1M PBS-
204 T. A Vectastain elite rabbit kit (Vector Laboratories; PK6101) was used to amplify the
205 antibody-antigen complex. The sections were then incubated for 60 minutes in biotinylated
206 goat anti-rabbit secondary antibody (1:250 in 0.1M PBS-T; Vector Laboratories), rinsed for
207 three 10-minute washes in 0.1M PBS-T and then incubated in 0.1M PBS-T for 60 minutes at
208 room temperature with avidin-biotin horseradish-peroxidase complex (ABC; Vector
209 Laboratories). Sections were then washed in three 10-minute washes in 0.1M PBS-T, and
210 then five minutes in 0.1M PBS. Sections were then briefly rinsed in 0.1M sodium acetate
211 buffer and developed with 0.04% nickel-intensified diaminobenzidene (Sigma) as the
212 chromagen for six minutes. To terminate the reaction, sections were rinsed a further six
213 times, each rinse lasting five minutes, in 0.1M PBS before being mounted with a paintbrush
214 onto gelatin coated slides, serially dehydrated through alcohol, cleared in xylene and cover-
215 slipped with glass coverslips using Pertex mounting medium (CellLife).

2.6. Fos immunoreactivity quantification

Fos-ir was quantified in the BSTmd and LS in the social behaviour network; the VTA in the dopaminergic reward/motivation circuit; and the AMV, AN, and ASt of the anterior motor pathway. These brain regions were selected as Fos-ir was previously reported to increase in these regions following nest building male zebra finches (Hall et al. 2014). Fos-ir was also quantified in all folia in the cerebellum. Areas of interest were located with reference to brain atlases of the canary (Stokes et al. 1974) and the zebra finch (Nixdorf-Bergweiler and Bischof 2007). To avoid any unconscious bias all slides were coded so the experimenter was unaware of the treatment group during Fos-ir quantification.

Images of each section were digitally captured using a Nikon E600 Brightfield Microscope camera and Zen 2 software, and stored on a laptop and server. See Table 1 for lens magnification. Each image was opened in ImageJ software version 1.5s (Schneider et al. 2012) and desaturated. *Auto levels* function was used to isolate Fos-ir nuclei from background staining. This function saturates the Fos-ir as black and the lack of Fos-ir as white. Before applying the function to each image, units were subtracted from the *auto levels* adjustment value (Table 1). The units subtracted differed between each brain region due to the variation in neuropil background staining, but were kept consistent for all samples within a region. After applying the *auto levels* function, the number of highlighted Fos-ir nuclei were counted in the image as a whole or in sub-sections (Table 1), either manually using a clicker or by using the *analyse particles* function in ImageJ (Table 1). Automatic counting was used for all regions apart from the BSTmd where the counting was manual. Nuclei were only counted if they fulfilled a predetermined criterion that differed with all brain regions due to the neuropil staining (Table 1). These criteria were selected by

measuring the area of the smallest Fos-ir nuclei identified in multiple, randomly selected sections across randomly selected birds. The number of suitable sections differed across the birds due to damage caused during sectioning and/or mounting of sections and therefore the number of Fos-ir nuclei in each section were summed and then averaged to yield a single value for each brain region in each bird.

Cerebellum sections were quantified live, using a Leica microscope with a video camera connection at x40 magnification with a 4.5 light intensity. Three sections for each male were selected and three circles (40.6 μm radius) placed semi-randomly on the molecular layer of each folia, with each circle touching at least one other. All Fos-ir cells (identified as a dark dot on the image) within the circles were counted manually and then averaged for each folia, for each male (Figure 1).

2.7. Statistical analysis

Hopping with string and *flying with string* were combined into one category – *carry*. *Hopping*, *jumping*, and *flying* were all combined into one category – *move,* for all three treatments. *Tugging while hopping* and *tugging while flying* were combined with *tugging*. We included the following behaviours in the analysis: *pick up*, *deposit, tuck, carry*, *tug, feeding* and *move*.

All statistical analyses were completed using R Studio (2012, ver 1.1.447) with R Development Core Team (2016, ver. 3.4.1) using packages '*plotrix'* (Lemon 2006), *'dplyr'* (Wickham et al. 2017), *'tidyr'* (Wickham and Henry 2009), and '*broom*' (Robinson and Hayes 2019). All graphs were created using '*ggplot2'* (Wickham 2009) '*cowplot'* (Wilke 2019), and *'ggsignif'* (Ahlmann-Eltze 2017). All means are shown and with standard errors. Behaviour and Fos-ir counts were compared as dependent variables using Generalised Linear Models

(GLM), with a negative binomial distribution using the '*MASS'* package (Venables and Ripley 2002), and the independent variable treatment on three levels (Building, Fixed, Control). Posthocs, using the '*multcomp*' package (Hothorn et al. 2008), were run on any forebrain region or cerebellum region that had differing Fos-ir levels. Type II likelihood-ratio chi-square tests ('*car'* package (Fox and Weisberg 2011)) were performed on all finalised GLMs to determine the significance of predictor variables.

To investigate whether behaviour explained individual variation in Fos-ir production GLMs with negative binomial distribution were run with Fos-ir counts as dependent variables and behaviour counts as independent variables. Only Building males were included in analyses with *deposit, carry* and *tuck*, while only Fixed males were included in analyses with *tug*. Both Building and Fixed males were included in behaviour analyses *move* and *feeding*, with behaviour counts*treatment as interactions. To account for the number of analyses conducted correlating Fos-ir and behaviours and the chance of including a Type I error, a sequential Bonferroni method was used (Holm 1979), adjusting the critical value for each model, by brain region and folia. The forebrain and the cerebellum were analysed separately.

3. Results

3.1. Behavioural analysis

In the 90-50 minutes prior to sacrifice, Control, Fixed and Building birds all moved around the cage to the same degree (GLM: χ^2_1 = 3.38, n = 26, p = 0.18). Control birds fed more than Building birds, while Fixed birds did not differ either of the other groups (GLM: χ^2_1 = 10.16, n = 16, p = 0.001). Building birds made more pick ups (GLM: χ^2_1 = 10.46, n = 26, p = 0.006; Building = 50 ± 16.23; Control = 178 ± 30.47; Building vs Control, p = 0.006).

288

289 *3.2. Forebrain*

290 In the anterior motor region, Building and Fixed males had more Fos-ir than did Control
291 birds in the AMV (GLM: χ^2_1 = 13.87, *n* = 24, *p* < 0.001; Control vs Fixed, p = 0.004; Control vs
292 Building, *p* < 0.001; Table 2; **Error! Reference source not found.**). In the AN and ASt it was
293 just the Building males that had more Fos-ir than did Control males (AN, GLM: χ^2_1 = 11.46, *n*
294 = 25, *p* = 0.003; Control vs Building, *p* = 0.002; ASt, GLM: χ^2_1 = 7.25, *n* = 25, *p* = 0.03; Control
295 vs Building, *p* = 0.01; Table 2; **Error! Reference source not found.**). The Fos-ir expression in
296 the AN and ASt of the Fixed birds did not differ from that in the Building or the Control
297 birds. Fos-ir in the LS was higher in Building compared to Fixed males, while Control Fos-ir in
298 the LS did not differ from that in Building or Fixed males (GLM: χ^2_1 = 7.85, *n* = 21, p = 0.02;
299 Building vs Fixed, *p* = 0.01; Table 2; **Error! Reference source not found.**). Fos-ir did not differ
300 by treatment in the BSTmd (GLM: χ^2_1 = 1.55, *n* = 21, p = 0.46; Table 2; **Error! Reference**
301 **source not found.**), or in the VTA (GLM: χ^2_1 = 0.64, *n* = 23, *p* = 0.69; Table 2; **Error!**
302 **Reference source not found.**).

303 There was increased Fos-ir expression in four regions of the forebrain in response to
304 handling of nest material. As the number of times Building males deposited material in the
305 nest increased, so did the amount of Fos-ir in the BSTmd, LS and AMV (Table 3; Figure 3).
306 Fos-ir also increased in the LS, AMV and ASt the more Building males carried material, while
307 an increase in tucking material in the nest correlated with an increase of Fos-ir in the LS and
308 the AMV (Table 3; Figure 3). As tugging of material in Fixed males increased, so too did Fos-
309 ir in the LS (Table 3; Figure 3). Neither variation in the number of times a bird picked up
310 material, nor in the number of times a bird moved or fed, explained variation in Fos-ir in any
311 of the brain areas (see Supplementary material).

3.3. Cerebellum

As Building males carried more material, Fos-ir increased in Folia VI (Table 4; Figure 4). There was increased Fos-ir in all cerebellar folia, except Folia VIII, as Fixed males tugged nest material (Table 4; Figure 4). As Building and Fixed males picked up more material, Fos-ir increased in Folia VIII and Folia X (Table 4; Figure 4) while as feeding increased, Fos-ir in Folia II, III, IV, V and VI decreased (Table 4; Figure 4). The number of times Building males deposited or tucked nest material did not explain variation in any folia, and moving did not explain folia Fos-ir variation in either Building or Fixed males. Finally, although Fos-ir differed by treatment in folium IX (GLM: $\chi^2_1 = 6.44$, $n = 22$, $p = 0.04$), posthoc testing showed no significant differences between the treatment groups.

4. Discussion

In the anterior motor pathway, nest-building males (Building) and males that could interact only with nest material that was tied down (Fixed) had more Fos-ir in the AMV than did males with no access to nest material (Control). Activation in the AMV increased as males deposited and tucked nest material and activation in the AMV and ASt increased as males carried nest material. Building males also had higher Fos-ir levels in the AN and ASt than did Control males, which indicates a role for the anterior motor pathway in nest building.

In the social behaviour network and dopaminergic reward circuitry, Fos-ir in the LS was higher in Building than in Fixed males and there was no difference in Fos-ir in the BSTmd and VTA between the three treatments. Fos-ir increased with depositing in the LS and BSTmd, while carrying, tucking and tugging of nest material caused activation in the LS.

335 In the cerebellum, Fos-ir differed by treatment only in folia IX, however activation in
336 nearly all folia was correlated with changes in behaviour. Fos-ir increased with tugging in all
337 folia (bar folia VIII), Fos-ir increased in folia VIII and X the more a male picked up nest
338 material and Fos-ir increased in folia II, III, IV, V and VI the more a male feed. Moving about
339 the cage did not account for neuronal activity in any of the forebrain regions or folia that we
340 measured.
341
342 *4.1. Forebrain*
343 There was greater activation in the all areas of the anterior motor pathway in Building birds
344 than Control birds, while Fixed birds had greater activation than Control birds only in the
345 AMV. As activation across the anterior motor pathway increased with nest-building
346 behaviours, these data establish the importance of the anterior motor pathway in nest
347 building. Furthermore, given the involvement of this motor pathway in motor learning and
348 sequencing (Feenders et al. 2008), these data are consistent with nest building being a
349 sequential behaviour that involves learning (Bailey et al. 2014; Breen et al. 2019; Muth and
350 Healy 2011; 2014; Walsh et al. 2013). Our data also support the suggestion of Hall et al.
351 (2014) that nest building may be underpinned by motor control similar to that which has
352 been recognised in tool use, in particular due to the increase activation in the ASt, an area
353 of the striatum active during tool use in both birds and mammals (Obayashi et al. 2001;
354 Reiner et al. 2004).
355 Our experimental design allowed us to make more specific associations between
356 activity in the different parts of the anterior motor pathway with the different building-
357 associated behaviours. In particular, although a role for the AMV and the ASt in nest
358 material collection (see Hall et al. (2014) is confirmed by the increase in activation in the

359 Building males the more they carried (AMV and ASt) and deposited (AMV) nest material, as
360 there was no difference in activation in these regions between Building and Fixed males and
361 no increase as males picked up material, regardless of treatment, they are unlikely to be
362 primarily regulating material collection. Indeed, as the activation in the AMV also increased
363 as males tugged at material that was tied down only in the Building birds (and not in the
364 Fixed birds), it looks as if the AMV is involved in nest building and not just in material
365 collection. Furthermore, as activation did not increase the more often Building and Fixed
366 males fed or moved, and this is a behaviour that uses similar muscle movements to those
367 used when the birds handle nest material, the activation of the AMV and ASt in association
368 with increased carrying, depositing and tucking of nest material seems unlikely to be due
369 just to the use of the neck, beak and wings muscles.

370 Activation levels in the BSTmd of Building males increased the more material a male
371 deposited in the nest, which fits with what we know about the role of the male in nest
372 building in zebra finches and that of the BSTmd in male sexual behaviour. In zebra finches it
373 is the male that selects material, carries it to the nestbox, and is typically the one to build
374 the nest (Zann 1996) and the increase in BSTmd Fos-ir in conjunction with increases in
375 depositing of material is also consistent with a role for the BSTmd in the neural control of
376 male courtship behaviours (e.g. Goodson 2005). One of those courtship behaviours could be
377 the possession of a nestbox (BSTmd activation increased in starlings that have a nestbox:
378 Heimovics and Riters 2006). But because in our study, the possession of a nestbox did not
379 affect the amount of Fos-ir in the Building and Fixed males relative to the Control birds,
380 which did not have a nest cup (as with Hall et al. 2014), it seems unlikely that BSTmd
381 activation in zebra finches is related to nestbox possession alone. Further work is required
382 to identify whether BSTmd activation occurred because males were engaging in nest

building, or because male zebra finches were performing a male sexual behaviour (Zann 1996). To determine whether the BSTmd is required for nest building rather than for a male sexual behaviour, it would be helpful to investigate activation levels in species where the female builds the nest. If BSTmd is active specifically in building, then one would expect activity in this region to be greater in nest-building females.

Activation in the LS was greater in Building than Fixed males, and increased the more Building males deposited, carried and tucked nest material and increased the more Fixed males tugged on tied down nest material. This finding corroborates the data of Heimovics and Riters (2006), which showed that the LS is activated as birds collect nest material (Heimovics and Riters 2005). As activation in the LS of our birds also increased with the number of times a bird tucked or tugged nest material, it may be related to interactions with nest material and not to nest building *per se* .

Finally, although we previously reported that activation in the VTA increased the more a Building male picked up material (Hall et al. 2014), we did not replicate that finding here. In the current study, VTA activation did not correlate with any behaviours (for neither Building nor Fixed males) analysed in this study. It is not clear why our data differ from those we reported in our previous study as the experiments were intentionally very similar.

4.2. Cerebellum

Differences in the degree to which the Fixed birds tugged at the nest material explained individual variation in Fos-ir expression in all folia, except for folia VIII. Tugging involved repetitive neck movements as males used their beak to pull at string that was tied down, and while doing so males also frequently hopped and flew around the string pile while tugging. This use of neck, leg, feet and wing muscles is probably the cause of activation in all

of these cerebellar folia. Folia I – VI receive projections from the brainstem and spinal divisions innervating neck musculature (Necker 2001), folium VI also receives input from leg, feet and wing muscles and folium IX receives input from the legs (Feenders et al. 2008). Fos-ir in folia II – VI also increased as the males fed, which suggests that while these folia are activated during material handling, they were not predominately activated because the birds were engaging in tugging nest material. Rather, it seems that similar neck movements are required to tug at material as to feed. Because tucking of nest material is a behaviour that seems to require similar neck musculature as to tugging, we might have expected tucking also to result in increased activation in these folia. But it did not, thus pointing to a need to look more closely at the muscle and bill movements required to build a nest, and how different use or degree of use of muscles activates different cerebellar folia.

Various movements explained in activation in folia VI, VII and X. Folium VI activity increased with the number of times males carried nest material, which is consistent with the stimulation of leg, feet and wing muscles (Feenders et al. 2008) while in folia VIII and X, activation increased as males picked up more nest material. Unlike the explicable relationship between movement and activation in folia, VI, why these activation in two folia should have increased with any motor output is not clear because these folia predominately receive visual information (Iwaniuk et al. 2007; Wylie et al. 2018). Perhaps picking up material requires visual perception in a clustered environment that enables detection and selection of desired material. But it is therefore surprising that tucking material in the nest, a behaviour that might also demand visual perception to ensure material is tucked in the correct location and manner, does not explain activation variation in folia VIII and X. Again, closer examination of the function of these folia is required to explain these behaviour-folia activation relationships.

5. Conclusion

By comparing neural activity in zebra finches that could build a nest (Building) and zebra finches that could only pick up and pull at material (Fixed), we have identified activity in the cerebellum, anterior motor pathway, social behaviour network and the dopaminergic reward circuitry that is specifically involved in the collection and/or handling of nest material in captive male zebra finches. Observing the occurrence of activation across these regions shows that nest building and material handling is more than just a series of fine-tuned motor actions.

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Figures

Figure 1. Drawing of a sagittal cerebellum section. Sampling protocol used to quantify Fos-ir in the molecular layer of the zebra finch cerebellum. Neurons with Fos-ir were live counted, with three sampling circles arranged in the molecular layer of the cerebellum in a chain of semi-random positions.

Figure 2. Mean number of Fos-ir nuclei in each forebrain region; A) AMV: anterior ventral mesopallium; B) AN: anterior nidopallium; C) ASt: anterior striatum; D) LS: lateral septum; E) BSTmd: bed nucleus of the stria terminalis, dorsal subdivision; F) VTA: ventral tegmental area. Sample sizes for each group are indicated at the bottom of each bar. Means and standard errors shown. * indicates significant differences (** $p > 0.001$; *** $p < 0.001$).

Figure 3: Correlations between nest building activities and Fos immunoreactivity in the bed nucleus of the stria terminalis (BSTmd), lateral septum (LS), anterior ventral mesopallium (AMV) and the anterior striatum (ASt). Within each graph the regression coefficient and *p* value are presented in the top-left corner. Graphs A-H represent Building males (filled circles) and Graph I represent Fixed males (open squares).

Figure 4. Correlations between nest building activities and Fos immunoreactivity in the cerebellum folia. Within each graph the regression coefficient and *p* value are presented in the top-left corner. Graphs A-I represent Fixed males (open squares) and Graphs J and K represent Building males (filled circles) and Graph L includes both Fixed (open squares) and Building (filled circles) males.

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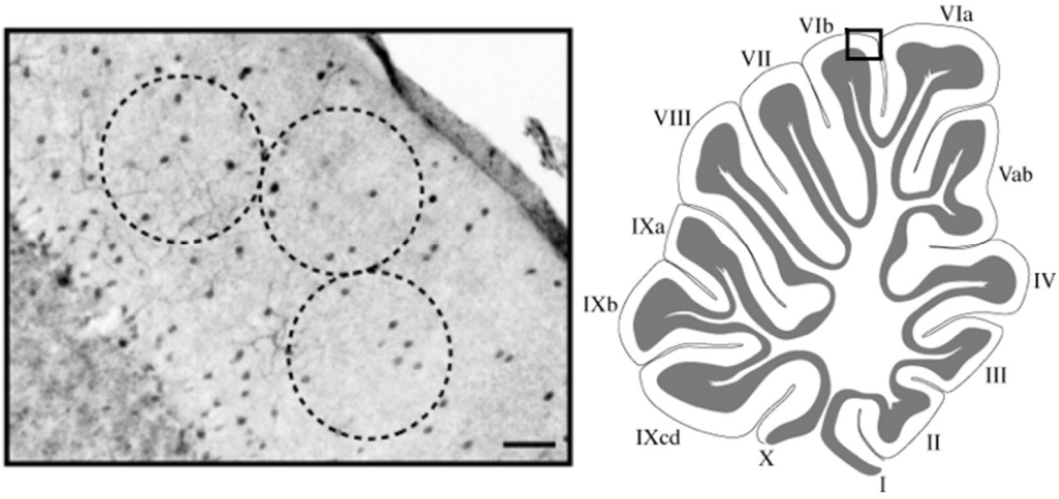

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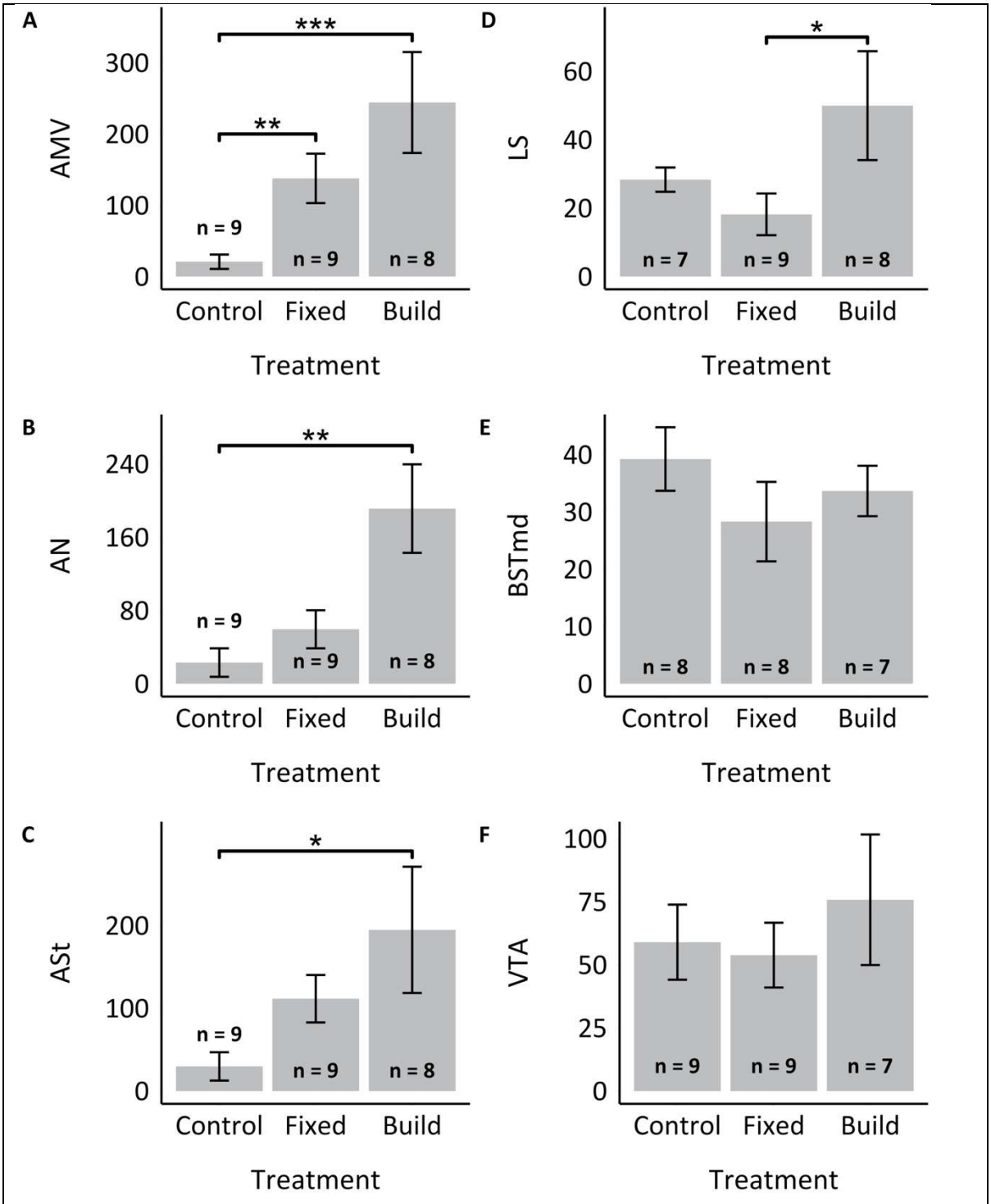

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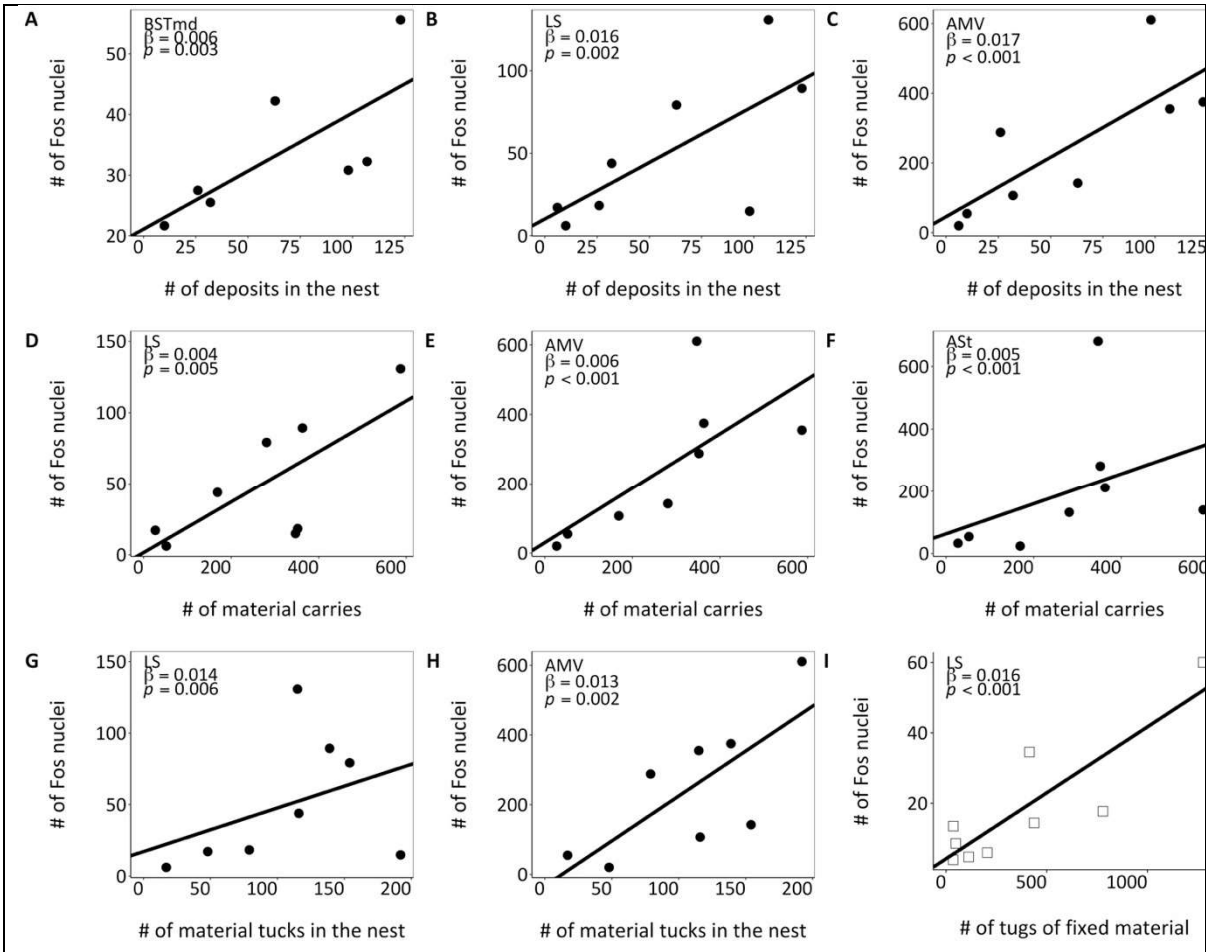

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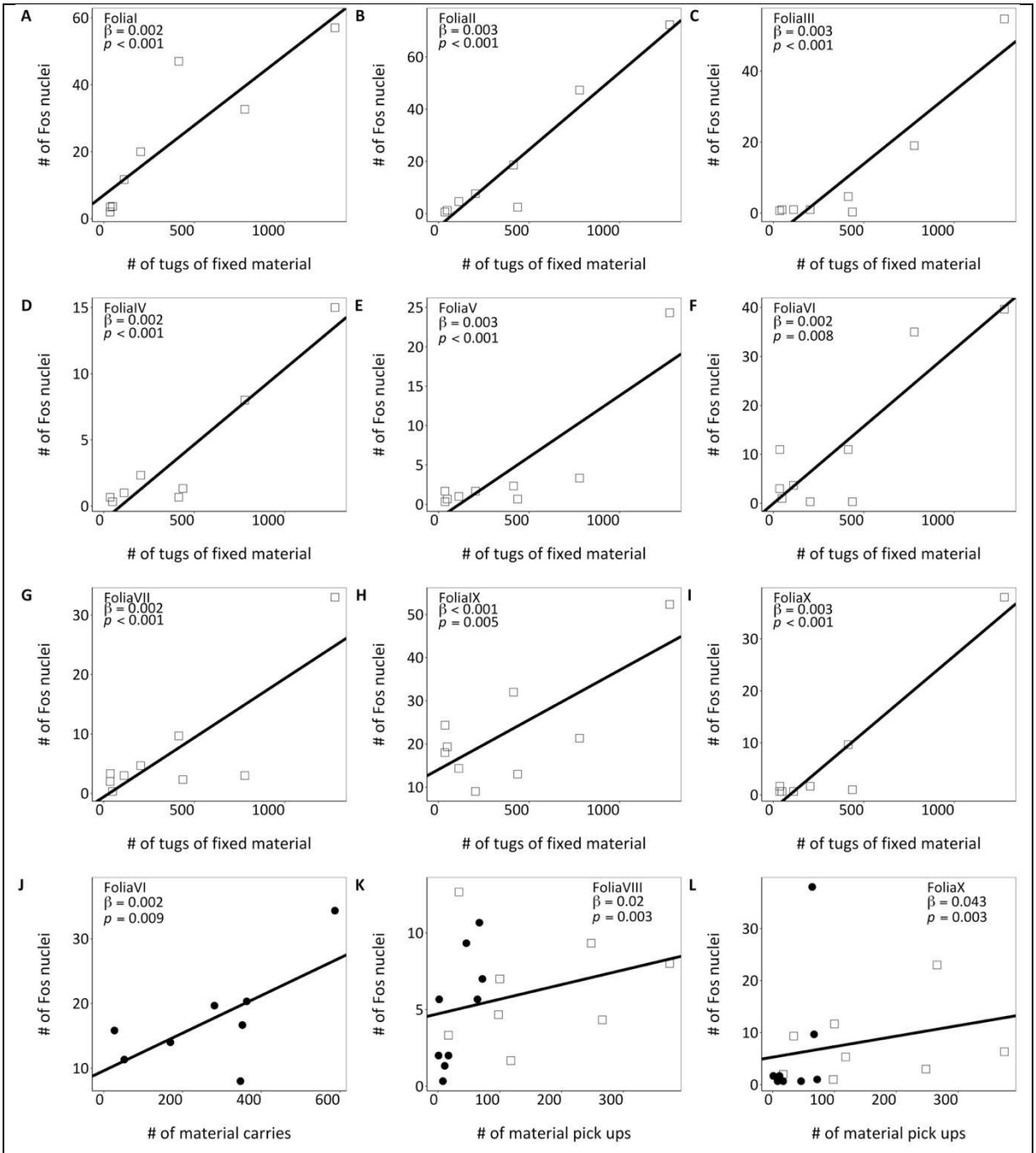

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Tables

Table 1. Criteria applied to each brain region for Fos-ir quantification. Each brain region required different measures due to the neurophil staining.

Table 2. Fos-ir means and standard errors for each of the three treatments for each brain region quantified.

Table 3. Behaviours that correlated with Fos-ir production in the brain regions of male adult zebra finches. As the listed behaviour increased, so did Fos-ir in the reported brain region. The Holm (1979) method was used to account for Type I errors. The critical value was set at 0.01. Carry, Tuck and Deposit analysis included only Building males, Tugging included only Fixed males, and Pick Up, Move and Feeding included both Building and Fixed males.

Table 4. Behaviours that correlate with Fos-ir production in the cerebellum folia of male adult zebra finches. +/- in the effect size column indicates the direction of Fos-ir in relation to behaviour. The Holm (1979) method was used to account for Type I errors. The critical value was set at 0.01. Carry, Tuck and Deposit analysis only included Building males, Tugging only included Fixed males and Pick Up, Move and Feeding included both Building and Fixed males.

Table 1 Criteria applied to each brain region for Fos-ir quantification. Each brain region required different measures due to the neurophil staining. BSTmd were manually counted due to the neurophil staining and ImageJ failing to detect cells stained for Fos-ir. This issue did not occur with other brain regions.

Region	**Objective lens (x)**	**Units subtracted from *auto levels* adjustment level**	**Count criteria**	**Whole image or sub sections sampled**
BSTmd	20	30	Manual count	Whole image
LS	10	25	*Analyse particles*: > 100 pixel count	3 circles (X pixel)
VTA	10	40	*Analyse particles:*150-800-pixel count	Whole image
AMV AN AST	10	30-40	*Analyse particles:*100-800-pixel count	Whole image

Table 2 Fos-ir means and standard errors for each of the three treatments for each brain region quantified.

		Means ± SE		
Brain region	Acronym	**Control**	**Fixed**	Building
Bed nucleus of the stria terminalis, dorsomedial subdivision	BSTmd	39.24 ± 5.55	28.31 ± 6.94	33.66 ± 4.40
Lateral Septum	LS	28.32 ± 3.55	18.17 ± 6.10	46.96 ± 15.92
Ventral Tegmental Area	VTA	58.97 ± 14.88	53.85 ± 12.83	75.78 ± 25.87
Anterior ventral mesopallium	AMV	20.72 ± 10.10	137.70 ± 34.68	244.21 ± 70.81
Anterior nidopallium	AN	23.13 ± 15.54	59.46 ± 20.78	191.20 ± 48.32
Anterior striatum	ASt	29.70 ± 17.14	111.52 ± 28.67	194.75 ± 76.30

Table 3. Behaviours that correlated with Fos-ir production in the brain regions of male adult zebra finches. As the listed behaviour increased, so did Fos-ir in the reported brain region. The Holm (1979) method was used to account for Type I errors. The critical value was set at 0.01. Carry, Tuck and Deposit analysis included only Building males, Tugging included only Fixed males, and Pick Up, Move and Feeding included both Building and Fixed males.

Brain Region	**Acronym**	**Behaviours**	***β***	***z***	***p value***
Bed nucleus of the stria terminalis, dorsomedial subdivision	BSTmd	Depositing	0.006	2.97	0.003
Lateral Septum	LS	Depositing	0.016	3.06	0.002
		Carry	0.004	2.78	0.005
		Tuck	0.014	2.75	0.006
		Tug	0.016	4.02	< 0.001
Anterior ventral mesopallium	AMV	Depositing	0.017	3.33	< 0.001
		Carry	0.006	5.22	< 0.001
		Tuck	0.013	3.11	0.002
Anterior striatum	ASt	Carry	0.005	3.33	< 0.001

Table 4. Behaviours that correlate with Fos-ir production in the cerebellum folia of male adult zebra finches. +/- in the effect size column indicates the direction of Fos-ir in relation to behaviour. The Holm (1979) method was used to account for Type I errors. The critical value was set at 0.01. Carry, Tuck and Deposit analysis only included Building males, Tugging only included Fixed males and Pick Up, Move and Feeding included both Building and Fixed males.

Folia	**Behaviours**	***β***	***z***	***p* value**
I	Tug	0.002	3.48	< 0.001
II	Tug	0.003	5.13	< 0.001
	Feeding	- 0.018	- 2.78	0.005
III	Tug	0.003	8.08	< 0.001
	Feeding	- 0.029	- 3.70	< 0.001
IV	Tug	0.002	5.78	< 0.001
	Feeding	- 0.024	- 4.21	< 0.001
V	Tug	0.003	7.28	< 0.001
	Feeding	- 0.022	- 3.21	0.001
VI	Carry	0.002	2.60	0.009
	Tug	0.002	2.67	0.008
	Feeding	- 0.014	- 2.48	0.01
VII	Tug	0.002	4.19	< 0.001
VIII	Pick up	0.020	2.92	0.003
IX	Tug	< 0.001	2.80	0.005
X	Pick up	0.043	3.01	0.003
	Tug	0.003	5.49	< 0.001